Improvement of an *invA*-based PCR for the specific detection of *Salmonella* Typhimurium in organs of pigs

Optimierung eines *invA* basierten PCR-Assays zur spezifischen Detektion von *Salmonella* Typhimurium in inneren Organen von Schweinen

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Summary:
The aim of this study was to investigate the suitability of the *invA*-based polymerase chain reaction (PCR) assay for the specific detection of *Salmonella* in organs of experimentally infected pigs and to compare these results to classical bacterial culture. While the PCR conditions specified in the "Deutsche Industrie Norm", DIN 10135 (§ 35 LMBG, 1999), revealed various unspecific amplification products, modifications of the PCR conditions allowed the specific amplification of the *invA* fragment from inner organs. The modified PCR assay correlates excellently with cultivation results (as required by DIN Norm 6579) and enables the detection of *Salmonella* within 48 hours with equal sensitivity compared to routine cultivation.

Keywords: Diagnostics, PCR, *invA*, experimental infection, inner organs

Introduction

The prevalence of *Salmonella* in German slaughtering pigs is estimated to be as high as 10.5 % depending on the method of investigation (Käsbohrer et al., 1997). Contaminated pork might represent an important source for human salmonellosis. In Germany 105.000 persons were infected with *Salmonella* in 1997, as stated by the Robert Koch Institute. Eighty-seven of these patients died. Beside this impact on human health, enormous financial losses might be caused by *Salmonella* infections worldwide. For these reasons, some European states e.g. Sweden and Denmark established control programs for the surveillance of *Salmonella* at the pre- and post harvest level. This includes controlling of feeds, nucleus and multiplier herds and monitoring of slaughtering pigs. The evidence of previous or indeed current infection may be identified rapidly by serology using an ELISA test on sera or meat juices (skirt muscle) to measure the level of anti-*Salmonella* antibodies. However, the drawback of this approach is that the assay does not

reflect the actual status of infection, but may represent an earlier contact with the pathogen. Therefore, this assay is only suitable for the screening at the herd level, but is not qualified to identify single infected animals. Conventional bacterial culturing of the pathogen by non-selective pre-enrichment, subsequent selective cultivation and biochemical identification is the most common method to detect *Salmonella* in routine diagnostics. Although, this method gives satisfying results the entire procedure is time-consuming, laborious and needs sophisticated laboratory procedures. Therefore, PCR assays have been developed in the last few years to rapidly and specifically detect *Salmonella* in faeces and inner organs of infected animals (Aabo et al., 1995; Chen et al., 1997; Amavisit et al, 2001).
The most critical step in a PCR is the presence of inhibitory substances in the sample matrix to be investigated (Amavisit et al., 2001; Chiu and Ou, 1996). Although, DNA preparation kits for the extraction of (bacterial) DNA from stool samples were developed (Qiagen, Invitek), the sensitivity is usually lower (when animal faeces are used) than that of traditional bacterial cultivation. Therefore, various efforts have been made to develop new, effective DNA extraction and pre-enrichment strategies prior to PCR.
In this study we have used the *invA* PCR assay as described in DIN 10135, which is based on the studies of Rahn et al. (1992) for the specific detection of *Salmonella* after non-selective and selective pre-enrichment. Embedded in an experimental *Salmonella* infection model in swine, we have evaluated this PCR assay for the detection of *Salmonella* in inner organs.
Here we demonstrate that a modified PCR protocol, but not the condition given in DIN 10135 are suitable to rapidly and specifically detect *Salmonella* in swine with a sensitivity comparable to conventional cultivation procedures.

Material and Methods

Specimen collection and selective enrichment of *Salmonella*

Fourteen specimens (palatine tonsils, mandibular lymph nodes (ln), lung, liver, spleen, jejunum, ileum, colon, caecum, lung ln, ileocaecal ln, jejunal ln, colic ln, and muscle) derived from pigs experimentally infected with *Salmonella* Typhimurium DT104 (for details see Marg et al., 2001) were investigated.
Specimens were processed for enrichment by the following procedures. One gram of each specimen was mixed with 9 ml of peptone-broth, homogenized using a Stomacher 400 (Seward, London, UK) at high speed for 2 min, and incubated at 37 °C for 24 hours. Then 0,1 ml were transferred into 10 ml of Rappaport-Vassiliadis medium, and incubated at 42 °C for further 24 hours (German DIN Norms 6579 and 10135). Aliquots of 1 ml were taken after 24 hours of peptone- and after 24 hours of Rappaport-Vassiliadis-enrichment for PCR analysis, respectively. To be able to compare the results obtained by PCR and conventional bacterial culturing,

an aliquot was subjected to conventional bacterial cultivation after selective enrichment.

Sample preparation for PCR

Crude tissue particles were removed by low-speed centrifugation (300 x g) for 3 min. After high-speed centrifugation (10,000 x g, 10 min) soluble substances were discarded with the supernatant and the pelleted bacteria were resuspended in 1 ml of PBS, and washed twice in PBS by repeated suspension and high-speed centrifugation steps. The final pellet was suspended in 200 µl lysis buffer containing a final concentration of 0.025 % Tween 20 (Sigma), and 0,2 mg/ml proteinase K (Merck), incubated for 1 h at 56°C, and heated to 95 °C for 10 min to inactivate the proteinase K. After centrifugation (13,000 x g, 5 min), 10 µl of the supernatant were subjected to PCR analysis.

Conditions for PCR experiments and electrophoresis

Ten µl of the template DNA were added to a mixture (40 µl) consisting of 5 µl 10x PCR-reaction-buffer with 15 mM $MgCl_2$ (MBI Fermentas, St. Leon-Rot, Germany), 5 µl 2mM dNTP mix (Prime Zyme), 10 pmol of each primer, 0,5 U *Taq* DNA polymerase (Roche, Mannheim). The primer pair ST-139 5′-GTGAAATTATCGCCACGTTCGGGCAA-3′ and ST-141 5′-TCATCGCACCGTCAAAGGAACC-3′ were used to amplify a 284 bp fragment within the conserved *invA* gene sequence of *Salmonella* spp., known to be specific for all species of the genus *Salmonella* (Rhan et al., 1992). Amplification was carried out in a GeneAmp PCR System 2400 (Perkin Elmer, Weiterstadt, Germany). Initially, the PCR conditions described by Rahn et al. (1992), were used (denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 53 °C for 2 min, and primer extension at 72 °C for 3 min).

This protocol was modified in order to obtain higher specificity. Optimization of cycling conditions resulted in an initial denaturation for 5 min at 94 °C followed by 35 cycles each consisting of 1 min denaturation at 94 °C, 30 sec annealing at 64 °C, and 30 sec extension at 72 °C, and a final extension step of 7 min. 5 µl of each PCR product were analyzed on a 2 % agarose gel at a constant voltage of 140 V in TAE buffer (40 mM Tris [pH 7.6], 20 mM acetic acid, and 1 mM EDTA). The PCR products were stained with ethidium bromide (0,5 µg per ml ddH_20) and were visualized under UV light by using the Quantity One®/Quantitation Software (Bio-Rad, Germany) from the Bio-Rad Gel Doc 2000 imager system.

Detection of *Salmonella* in various inner organs by PCR

Using the PCR conditions described by Rahn et al. (1992) various unspecific amplification products (> 284 bp) were obtained (data not shown). After optimization of the cycle parameters by increasing the annealing temperature (from 53 °C to 64 °C) and reducing the extension time (from 3 min to 30 sec) and the annealing time (from 2 min to 30 sec), the expected and specific PCR fragment of 284 bp of the *invA* gene was amplified from the investigated inner organs (Figure 1, arrow).

PCR was carried out after 24 hours of non-selective (Figure 1, A) and additional 24 hours of selective enrichment (Figure 1, B). An example for the amplification (one animal) obtained after 24 and 48 hours is shown in Figure 1. After 24 hours only specifically contaminated organs gave a positive PCR reaction, whereas after additional selective enrichment, the results did highly correlate with bacterial cultivation (data not shown). In most of the cases, *Salmonella* was detected from tonsils (Figure1, lane 3) and the intestine and its associated lymph nodes. However, from tonsils of four animals no PCR products were obtained, but these pigs were diagnosed positive when intestinal lymph nodes were used as template. These results were also confirmed by bacterial examination (data not shown).

Discussion

The PCR conditions given in DIN 10135 are based on a publication by Rahn et al. (1992) and have been evaluated by using single colonies of pure cultures of different *Salmonella* serovars and other *Enterobacteriaceae* as template for the PCR.

In practice, our experience is that unspecific side-products or failures due to inhibitory substances do occur when samples like faeces or inner organs are used for PCR. Therefore, the aim of this study was to evaluate the *invA*-based PCR assay for the detection of *Salmonella* in inner organs of pigs experimentally infected with *Salmonella* Typhimurium. After modification of the cycle conditions, the *invA* fragment could be specifically amplified from all investigated organs. This demonstrates that this modified DIN PCR assay evaluated for pure cultures is also suitable for the specific detection of *Salmonella* in inner organs of pigs. However, without pre-enrichment *Salmonella* could not be detected. Therefore, pre-enrichment is required to detect *Salmonella.* The comparison of culture and the PCR assay revealed the same sensitivity by PCR after 48 hours. In few cases, *Salmonella* could be detected by PCR but not by culture (data not shown). The results obtained after 24 hours of non-selective enrichment show a significant lower detection rate of *Salmonella* than bacterial cultivation.

Based on our results we recommend this modified *invA* PCR assay for the specific detection of *Salmonella* in inner organs of pigs. A combination of tonsil tissue and

tissue of intestinal lymph nodes (iliocaecal and jejunal lymph nodes) should be used for sampling and subsequent detection.

Acknowledgements

This work was supported, in parts, by the Deutsche Forschungsgemeinschaft (DFG, GRK-39/2). Heike Marg and Thorsten Arnold acknowledge the DFG for a predoctoral fellowship (Graduiertenkolleg).

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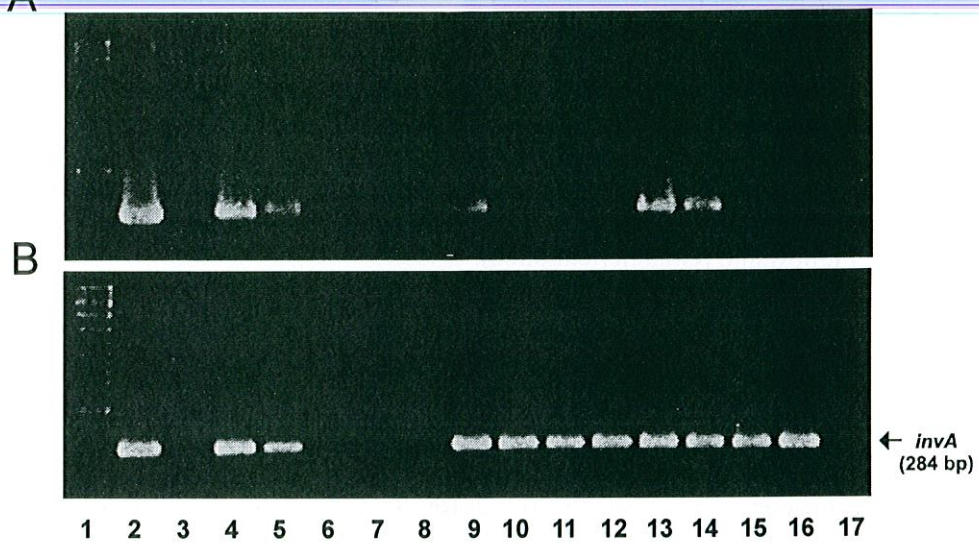

Figure 1
An *invA* PCR to detect *Salmonella* in inner organs of pigs. The positive amplification of the 284 bp *invA* fragment is indicated by an arrow. **A**, after 24 hours of non-selective enrichment. **B**, after additional 24 h of selective enrichment. Lane 1, molecular weight standard; lane 2, positive control; lane 3, negative control. Lane 4 – 17, inner organs in the following order: tonsils, mandibular lymph nodes (ln), lung, liver, spleen, jejunum, ileum, colon, caecum, lung ln, ileocaecal ln, jejunal ln, colic ln, and muscle.